albicans strains adhere better to epithelial cells of the oral cavity or the esophagus, but it is accepted that esophageal candidiasis represents a form of invasive candidiasis which requires higher dosages of antifungal drugs compared to OPC [11]. In this patient, replacement of a fluconazole-resistant *C. albicans* strain by a genetically different fluconazole-sensitive strain was observed; this finally caused further recurrences of OPC, which responded well to intermittent therapy with itraconazole solution. However, it is unclear whether the disappearance of the resistant genotype is due to the temporary increase of the CD4 cell count or because of therapy with itraconazole despite high-level resistance to azoles in vitro.

It may be concluded that serial DNA typing of *C. albicans* strains, together with antifungal susceptibility testing, may help us to monitor the susceptibility of clinical isolates to azole therapy.

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Detection of *Legionella pneumophila* DNA in urine and serum samples from patients with pneumonia

P. Matsiota-Bernard[1]*, S. Waser*[1] *and G. Vrioni*[1,2]

[1]Laboratoire de Microbiologie, Hôpital Raymond Poicaré, Faculté de Médecine de Paris-Ouest, Université Paris V, 92380 Garches, France and [2]Microbiology Laboratory, KAT Hospital, Athens, Greece

*Tel/Fax: +30 1 77 85 638 E-mail: pmatsiotabernard@internet.gr

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Many *Legionella* species are found in the natural environment and one of them, *Legionella pneumophila*, is responsible for more than 90% of cases of Legionnaires' disease [1]. Outbreaks of community-acquired and nosocomial *L. pneumophila* infections have been described [2,3].

The laboratory detection of the *Legionella* infection remains difficult. The serological diagnosis, although fairly specific, has a sensitivity in the 70–80% range depending on the following: (i) the single antibody test used ,and (ii) the detection of antibody to *Legionella* species or serogroups other than *L. pneumophila* serogroup 1 [4]. The results are also highly influenced by the kind of antigen (formolized or heated) used for the diagnosis of seroconversion. In addition, serological diagnosis can be difficult since the rise in antibody titres can be delayed.

The sensitivity of the direct fluorescence assay for the antigen detection remains highly variable, from 30 to 80% [5]. The detection of the *L. pneumophila* serogroup 1 urinary antigen with commercially available enzyme immunoassay (EIA) kits

seems highly specific (100%) and fairly sensitive (percentages vary from 63.76 to 66.66% in non-concentrated urine and from 86.66 to 88.88% in concentrated urine) [6]. However, the urinary kits are expensive and the need to include control tests increases their cost, particularly for small series.

The culture of the bacterium remains the standard and probably continues to be the most sensitive means of diagnosis when it is performed early in the course of disease. However, the isolation of *L. pneumophila* is time-consuming and needs special culture media. Moreover, it has been reported that several *L. pneumophila* strains grown in chick embryos, are not detectable by culture on agar media [7].

DNA amplification is an interesting alternative method for bacterial detection. Amplification of *Legionella*-specific DNA sequences has been used to detect the bacterium in environmental water samples [8,9]. Primers selected from the macrophage infectivity potentiator gene (*mip*) of *L. pneumophila* [10] have also been used for detection in environmental water and, more recently, in bronchoalveolar lavage fluid [11]. We described the use of a commercial kit, which was designed to detect the DNA of both the genus *Legionella* and the species *L. pneumophila* in environmental water samples, for the detection of *L. pneumophila* in clinical samples [12]. More recently, Murdoch et al. [13] described the detection of *Legionella* DNA in urine and serum samples, from patients with pneumonia, with a polymerase chain reaction (PCR) assay by using two 20-mer primers (L5SL9 and L5SR93), which amplified a 104-bp segment of the coding region of the 5S rRNA gene.

The aim of the present study was the detection of *L. pneumophila* DNA with a PCR and microplate hybridization assay in serum and urine samples from patients with legionellosis.

Serum samples from 41 patients with proven legionellosis and from 10 patients with pneumonia due to other organisms (controls) were studied. The legionellosis was proven either by positive *L. pneumophila* culture (nine patients), or by *L. pneumophila* antibody detection: if we had only one serum the antibody titre was more than 256, and if we had two contiguous samples (second sample taken 15 days later) from the same patient, the second one had a rise in antibody titre of more than four-fold.

Thirty samples of urine from the same set of patients with pneumonia (we had sera not accompanied by urine) were also tested for the presence of *L. pneumophila* serogroup 1 antigen with the Binax (Portland, ME, USA) enzyme immunoassay and 20 samples that were found to be positive and 10 that were found to be negative were included in the study.

Total DNA from the serum samples was extracted using a DNA extraction reagent containing the Chelex anion-exchange resin (Perkin Elmer, NJ, USA), as described previously [14]. Total DNA from the urine samples was extracted using the IsoQuick reagent (ORCA Research Inc., Bothell, WA, USA) [15–17].

A PCR assay with primers Leg1 (5′-GTCATGAGGAATCTCGCG-3′) and Leg2 (5′-CTGGCTTCTTCCAGCTTCA-3′) that specifically amplify a 700-base pair fragment of *L. pneumophila* was used as described previously [18], in order to detect the DNA of this bacterium in serum and urine samples from patients with pneumonia. The amplification products were at first visualized after agarose-gel electrophoresis and ethidium bromide staining.

To enhance the sensitivity and specificity of the PCR assay, amplified products were detected after hybridization in Hybridowell microtitre plates (Argene Biosoft, Varilhes, France), according to the manufacturer's instructions. Briefly, 2 μl of amplified product was directly coated on a microtitre plate, and incubated for 2 h at 37 °C. After hybridization with hybridization solution containing 50 ng/mL of the biotinylated oligonucleotidic probe Leg3 (5′-GTCCGTTATGGGGTATTGATCACC-3′) [18] for 30 min at 37 °C, hybrids were detected by streptavidin-peroxydase conjugate. The chromogen used was tetramethylbenzidine and the instrument used to monitor the signal was a microwell plate reader. The cut-off (CO) was calculated from the mean of the two values obtained with the kit negative control according to the equations: OD reading at 450 nm: CO = OD mean (negative control) $\pm$ 0.150 and OD reading at 450/650 nm: CO = OD average (negative control) + 0.075.

The negative control OD of the kit must be $<$0.4 OD units. The OD obtained with the amplification positive control must be above the cut-off. If the controls are validated, the results may be analyzed as follows: if the OD $>$ CO + 10%; the amplification is positive, if the OD $>$ CO $-$ 10%; the amplification is negative and if the OD = CO $\pm$ 10%; the amplified product must be retested.

Sequencing (ABI PRISM dye-terminator cycle sequencing) of three of the amplification products was used to confirm the specificity of the method.

Legionella pneumophila DNA was detected in 12 serum samples from the 41 patients with legionellosis (29%) and it was not detected in serum samples from the 10 controls. The presence of *L. pneumophila* DNA in the serum samples was not found to be related to the presence of positive cultures from bronchoalveolar aspirates or the serum antibody titres. In addition, positive results were found both in seven of the 15 'early' (2–4 days) and in five of the 26 'late' (10–15 days) serum samples after the onset of pneumonia. *Legionella pneumophila* DNA was also detected in six urine samples from the 20 patients with legionellosis and it was not detected in urine samples from the 10 controls. Positive results were found both in two of nine 'early' and in four of 11 'late' urine samples after the onset of pneumonia, as described in serum samples.

Our results are in accordance with those previously reported by Murdoch et al. [13], which described the detection of *Legionella* DNA by PCR in urine and serum samples from

patients with pneumonia. These authors used primers that amplified a segment of the 5S rRNA gene present in all *Legionella* species followed by a restriction enzyme analysis to confirm the specificity of the amplified PCR products. We obtained in our study a relatively lower sensitivity in sera and especially in urine samples although the samples tested were obtained from patients with *L. pneumophila* serogroup 1 infection and the primers used were specific for the bacterium.

The detection of *Legionella* DNA in the serum and urine samples remains a good alternative as diagnosis by the culture of the bacterium is time-consuming and needs special culture media and serological diagnosis is not very sensitive. Our results suggest that the molecular methods we used were specific but not very sensitive. In order to improve the sensitivity of our methods we must try other DNA extraction methods and primers and probes from different sequence regions. Indeed, Murdoch et al. [13] obtained a better sensitivity by using another amplification and hybridization target. The DNA concentration from urine could also play a role in the sensitivity of the method, as described for urinary antigen detection by EIA [6], however, the PCR before hybridization increases the DNA concentration in the sample examined. Collection of urine samples on consecutive days could increase the sensitivity of the method, since excretion of DNA in urine can be intermittent as described by Murdoch et al. [13]. Finally, the relatively low frequency of the bacteremia associated with legionellosis, in addition to the small amount of breakdown of nucleic acid products after macrophage lysis, could explain the poor sensitivity of DNA detection in the serum.

In conclusion, our results suggest that the detection of *L. pneumophila* DNA by our method can assist the rapid diagnosis of *Legionella* infection. Further investigations will be needed to see if the sensitivity could be increased by testing multiple specimens, by using a modification of the assay or other PCR targets and assays. Whether positive results are due to *Legionella* bacteremia or to breakdown products (nucleic acids) that are present during the infection, also remains to be elucidated.

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